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Alleles of the homologous recombination gene, *RAD59*, identify multiple responses to disrupted DNA replication in *Saccharomyces cerevisiae*

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Abstract

Background: In *Saccharomyces cerevisiae*, Rad59 is required for multiple homologous recombination mechanisms and viability in DNA replication-defective *rad27* mutant cells. Recently, four *rad59* missense alleles were found to have distinct effects on homologous recombination that are consistent with separation-of-function mutations. The *rad59-K166A* allele alters an amino acid in a conserved α-helical domain, and, like the *rad59* null allele diminishes association of Rad52 with double-strand breaks. The *rad59-K174A* and *rad59-F180A* alleles alter amino acids in the same domain and have genetically similar effects on homologous recombination. The *rad59-Y92A* allele alters a conserved amino acid in a separate domain, has genetically distinct effects on homologous recombination, and does not diminish association of Rad52 with double-strand breaks.

Results: In this study, *rad59* mutant strains were crossed with a *rad27* null mutant to examine the effects of the *rad59* alleles on the link between viability, growth and the stimulation of homologous recombination in replication-defective cells. Like the *rad59* null allele, *rad59-K166A* was synthetically lethal in combination with *rad27*. The *rad59-K174A* and *rad59-F180A* alleles were not synthetically lethal in combination with *rad27,* had effects on growth that coincided with decreased ectopic gene conversion, but did not affect mutation, unequal sister-chromatid recombination, or loss of heterozygosity. The *rad59-Y92A* allele was not synthetically lethal when combined with *rad27*, stimulated ectopic gene conversion and heteroallelic recombination independently from *rad27*, and was mutually epistatic with *srs2*. Unlike *rad27*, the stimulatory effect of *rad59-Y92A* on homologous recombination was not accompanied by effects on growth rate, cell cycle distribution, mutation, unequal sister-chromatid recombination, or loss of heterozygosity.

Conclusions: The synthetic lethality conferred by *rad59* null and *rad59-K166A* alleles correlates with their inhibitory effect on association of Rad52 with double-strand breaks, suggesting that this may be essential for rescuing replication lesions in *rad27* mutant cells. The *rad59-K174A* and *rad59-F180A* alleles may fractionally reduce this same function, which proportionally reduced repair of replication lesions by homologous recombination and growth rate. In contrast, *rad59-Y92A* stimulates homologous recombination, perhaps by affecting association of replication lesions with the Rad51 recombinase. This suggests that Rad59 influences the rescue of replication lesions by multiple recombination factors.

Keywords: Homologous recombination, *Saccharomyces cerevisiae*, DNA replication, Genome stability, Loss of heterozygosity

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Background

In *Saccharomyces cerevisiae*, defective DNA replication stimulates homologous recombination (HR), suggesting that the lesions that accumulate following replication failure are substrates for HR [1-11]. Rad27 is a structure-specific endonuclease [12] required for completion of lagging strand synthesis [13], and has also been implicated in base excision repair [14], and double-strand break repair by non-homologous end joining [15]. Loss of Rad27 leads to accumulation of single-stranded gaps or nicks on daughter DNA strands [2,16]. Collision of replication forks with these lesions results in fork collapse and generation of double-strand breaks (DSB) [8,17] that can stimulate HR. Importantly, concomitant loss of Rad27 and components of the HR apparatus leads to synthetic lethality [18-20]. These observations implicate HR in repair of DSBs that accumulate in the absence of Rad27. Failure to repair DSBs leads to chromosome loss [21] that is greatly stimulated in *rad27* null mutant cells [8], suggesting that the essential role for the HR apparatus in *rad27* mutants may be prevention of lethal levels of chromosome loss.

RAD59 encodes a protein that augments the ability of Rad52, the central HR protein in yeast [22,23], to anneal complementary DNA strands *in vitro* [24], and both are required for viability in *rad27* null mutant cells [19,20]. *RAD59* and *RAD52* are also required to repair DSBs by single-strand annealing (SSA) [21,25-28], and HR between inverted repeats by an annealing-dependent template switch at stalled replication forks [29-31]. Since *RAD59* exerts much of its effect on HR with *RAD52* [21,32,33], the function of *RAD59* required in the absence of *RAD27* may be in collaboration with *RAD52*.

The purpose of the current study was to explore the function of *RAD59* required for the viability of *rad27* null mutant cells. We investigated how four *rad59* mutations previously characterized with respect to their effects on SSA [21,27], affected survivorship when combined with a *rad27* null mutation. We found that *rad59-K166A*, which alters an amino acid in a conserved, putative α-helical domain [27,34,35], was synthetically lethal in combination with *rad27*. Because *rad59-K166A* diminishes association of Rad52 with DSBs [21], this may be a function required for the viability of *rad27* null mutant cells. The *rad59-K174A* and *rad59-F180A* mutations, which alter amino acids in the same α-helical domain, and have genetically similar effects on SSA [21], were not synthetically lethal with *rad27*, but resulted in distinct effects on growth that correlated with their degree of inhibition of HR. This strongly implicates *RAD59*-dependent HR as a requirement for viability in *rad27* null mutant cells. The *rad59-Y92A* mutation, which alters an amino acid in a separate, conserved loop domain and confers genetically distinct effects on SSA [27,34] was not synthetically lethal with *rad27*, and had a stimulatory effect on HR. This effect was genetically equivalent to that of a null allele of *SRS2*, which encodes a helicase that disassembles Rad51-DNA filaments [36,37], suggesting that Rad59 may affect association of Rad51 with replication lesions. The distinct effects of the *rad59* alleles suggest that Rad59 possesses multiple, discrete roles in responding to the consequences of dysfunctional replication.

Results

The *rad59* mutant alleles display distinct effects on survival and growth in cells defective for lagging strand synthesis

To further explore the function of *RAD59* required for viability in *rad27* null mutant cells, the effects of combining the *rad27::LEU2* allele with the various *rad59* alleles were determined by examining their ability to yield viable spores upon co-segregation in genetic crosses. The various *RAD27/rad27::LEU2 RAD59/rad59* double heterozygotes were sporulated and tetrads dissected onto rich medium (Figure 1). As observed previously, the *rad27::LEU2* and *rad59::LEU2* alleles did not appear together in any of the colonies arising from the spores, consistent with synthetic lethality [19,20]. The *rad59-K166A* allele, which alters a conserved lysine in the region of Rad59 that corresponds to the α-helical domain of the β – β – β – α motif of human Rad52 (Additional file 1: Figure S1) [27,34,35] displayed the same failure to appear with the *rad27::LEU2* allele, indicative of synthetic lethality.

The *rad59-K174A* and *rad59-F180A* alleles alter conserved amino acids in the same putative α-helical domain as *rad59-K166A* but were able to form viable spores upon segregation with *rad27::LEU2* (Figure 1). Doubling time of the *rad27::LEU2 rad59-F180A* double mutant was a statistically significant ($p = 0.045$) 24% longer than that observed for the *rad27* single mutant, which correlated with a ratio of G1 to S + G2/M cells that was a statistically significant ($p = 0.0031$) 2.6-fold lower (Figure 2; Additional file 1: Table S2). In contrast, doubling time of the *rad27::LEU2 rad59-K174A* double mutant was not significantly different from that of the *rad27::LEU2* single mutant ($p = 0.71$) (Table 1; Additional file 1: Table S2).

The *rad59-Y92A* allele alters a conserved amino acid in another region of extensive conservation with Rad52 (Additional file 1: Figure S1) [27,34], and was observed to yield viable spores upon segregation with *rad27::LEU2* (Figure 1). While the colonies derived from the *rad27::LEU2 rad59-Y92A* double mutant spores sometimes appeared smaller than the *rad27::LEU2* single mutant colonies on dissection plates, neither the doubling times ($p = 0.707$) (Table 1; Additional file 1: Table S2), nor the ratios of G1 to S + G2/M cells ($p = 0.60$) (Figure 2, Additional file 1: Table S2) were significantly different for the *rad27::LEU2* single and *rad27::LEU2 rad59-Y92A*

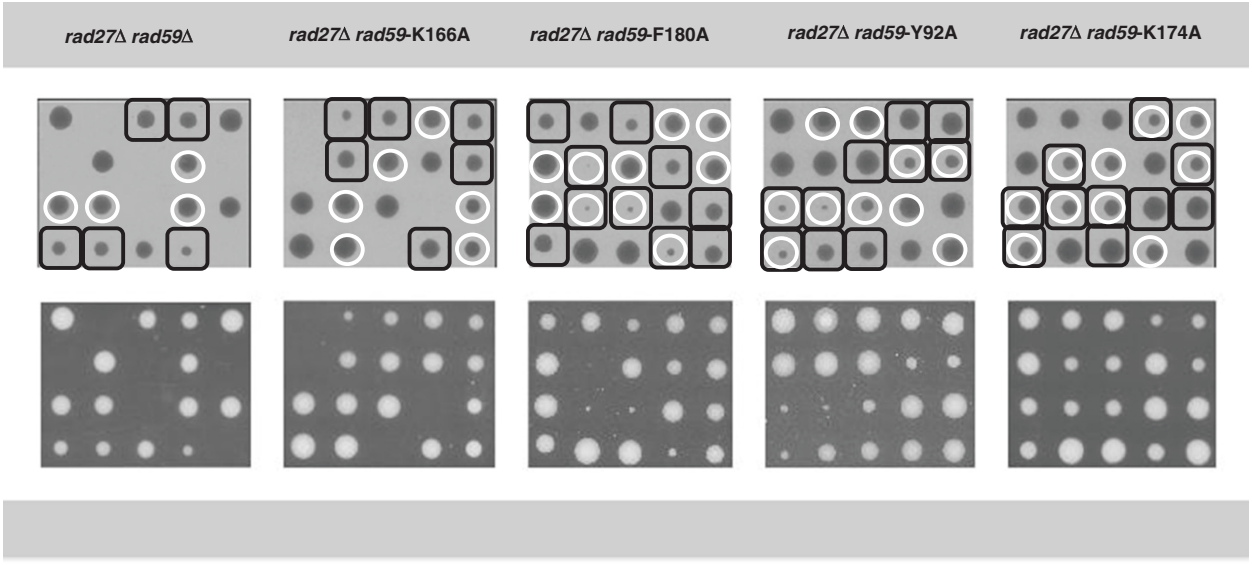

Figure 1 The *rad59* mutant alleles have distinct effects on survival in cells that are defective for lagging strand synthesis. Diploid strains heterozygous at the *RAD27* (*rad27::LEU2/RAD27*) and *RAD59* (*rad59/RAD59*) loci were sporulated and tetrads dissected onto YPD medium. The resulting colonies were examined after 72 h of growth at 30°. Colonies from five representative tetrads from each strain are displayed. The genotype of each colony was determined by PCR as described in the Methods. In the inverted image, colonies possessing a *rad27::LEU2* allele are boxed in black, and those possessing a *rad59* allele are circled in white.

double mutant strains. This suggests that germination of *rad27::LEU2 rad59-Y92A* double mutant spores may sometimes take longer than *rad27::LEU2* single mutant spores. We did not observe significant effects of the tested *rad59* missense alleles on doubling time ($p > 0.15$) (Table 1; Additional file 1: Table S2), or cell cycle distribution ($p > 0.50$) (Figure 2; Additional file 1: Table S2) in cells that possessed a wild-type *RAD27* gene. Since all four *rad59* missense mutations support steady-state levels of Rad59 that are comparable to wild-type [27], their effects on viability and growth when combined with *rad27::LEU2* cannot be attributed to changes in the level of Rad59 in the cell. Altogether, these observations suggest that *RAD59* plays a critical role in determining the growth characteristics of cells defective for lagging strand synthesis.

The *rad59* alleles affect a *RAD51*-dependent mechanism for repairing replication lesions

The central strand exchange factor, Rad51 [38], is often required for mechanisms of HR that require *RAD59*, including those involved in spontaneous HR between inverted and unlinked repeat sequences [39,40]. Like *RAD59*, an intact *RAD51* gene is necessary for viability in *rad27::LEU2* mutant cells [18-20], suggesting that *RAD51*-dependent HR plays a critical role in responding to replication lesions. Accordingly, loss of *RAD27* results in increases in HR events that require *RAD51* [18]. We used an assay that measures spontaneous ectopic gene conversion involving unlinked, mutant alleles of the *SAM1* gene [41] to examine effects of the *rad27::LEU2* mutation on HR in haploid strains (Figure 3A). Loss of *RAD27* resulted in a dramatic, 4,700-fold increased rate of ectopic gene conversion (Figure 3B; Additional file 1: Table S2), indicating that accumulation of replication lesions can greatly stimulate HR between unlinked sequences.

The robust stimulatory effect of the loss of the *RAD27* gene on ectopic gene conversion suggested that it could be used for examining the relationship between HR, and growth in the viable *rad27 rad59* double mutants. As observed previously [40], the *rad59::LEU2* mutation conferred a statistically significant 2.7-fold reduction in the rate of ectopic gene conversion (Figure 3B; Additional file 1: Table S2), confirming that *RAD59* plays a role in spontaneous HR between unlinked repeats. While neither the *rad59-K174A* nor *rad59-F180A* mutations had significant effects on their own, they led to significant, 3.1- and 9.3-fold reductions in the stimulatory effect of the *rad27::LEU2* allele in the *rad27::LEU2 rad59-K174A* and *rad27::LEU2 rad59-F180A* double mutants (Figure 3C; Additional file 1: Table S2), suggesting that they confer defects in the utilization of replication lesions by HR.

In contrast to the *rad59-K174A* and *rad59-F180A* mutations, the *rad59-Y92A* mutation caused an 86-fold increased rate of spontaneous ectopic gene conversion (Figure 3B; Additional file 1: Table S2), and, when combined with the *rad27::LEU2* mutation, stimulated the rate of ectopic gene conversion by a statistically significant 7.7-fold over that observed in the *rad27::LEU2*

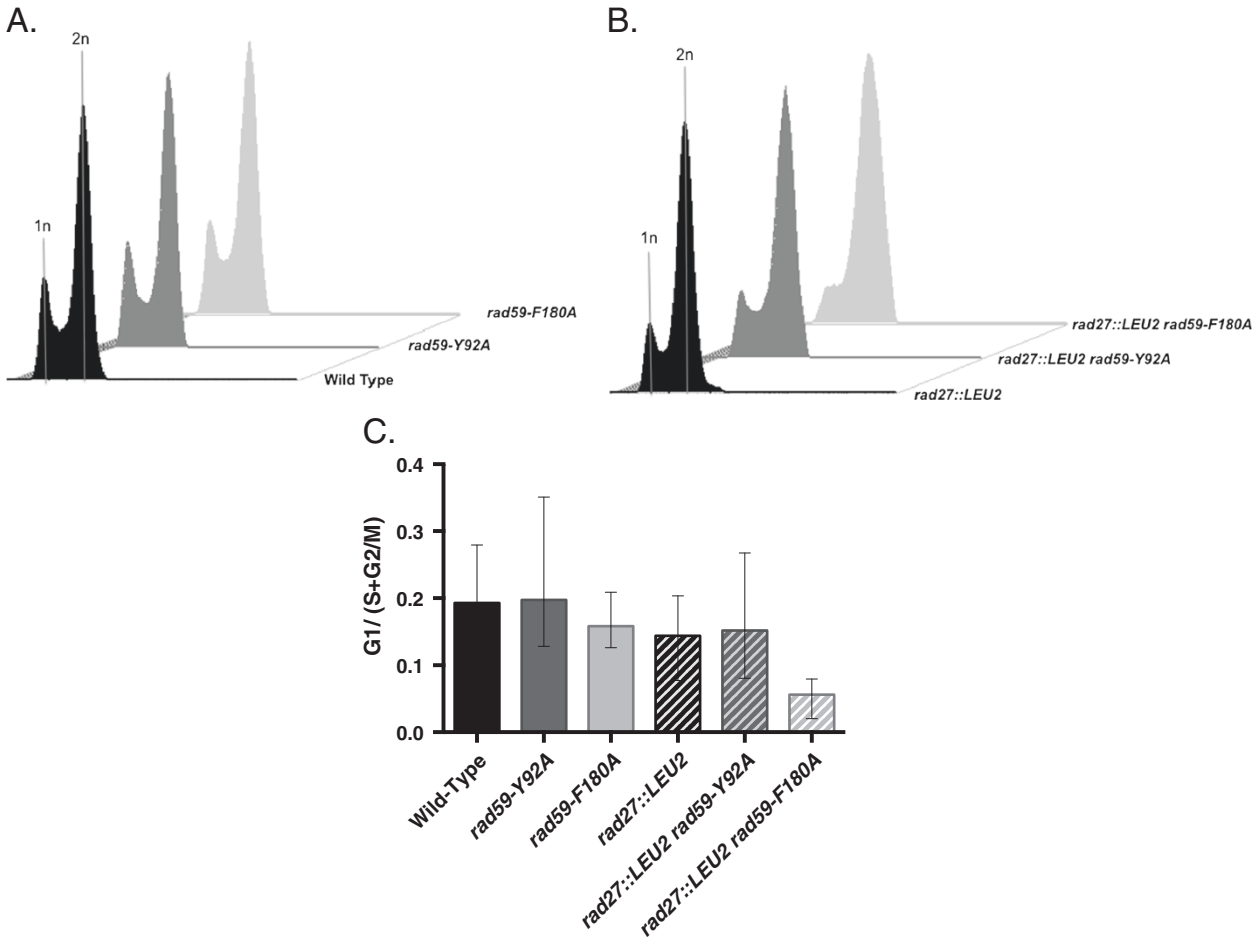

Figure 2 The *rad59* mutant alleles have distinct effects on cell cycle distribution in *rad27::LEU2* mutant cells. Wild-type, single and double mutant strains were grown to mid-log phase at 30°, fixed, stained with propidium iodide, and submitted to flow cytometric analysis as described in the Methods. **(A)** Cell cycle profiles for wild-type and *rad59* mutant strains. **(B)** Cell cycle profiles for *rad27* single and *rad27 rad59* double mutants. The distribution of cells with 1n and 2n DNA content in representative cultures of each strain are depicted. **(C)** Cell cycle distribution for wild-type and mutant strains. Median ratios of G1 to S + G2/M cells from a minimum of five independent cultures are indicated for each strain, and 95% confidence intervals are plotted.

Table 1 Doubling times in wild-type and mutant haploid cells

Genotype	Doubling time (min)	95% confidence interval
Wild-type	111	99, 120
rad59-Y92A	119	97, 124
rad59-K174A	131	111, 147
rad59-F180A	112	99, 128
rad27::LEU2	164	137, 180
rad27::LEU2 rad59-Y92A	176	136, 195
rad27::LEU2 rad59-K174A	153	126, 177
rad27::LEU2 rad59-F180A	205	183, 230

Doubling times of freshly dissected segregants were determined as described in the Methods. Displayed for each genotype is the median doubling time and 95% confidence interval, determined from at least ten independent cultures.

single mutant (Figure 3B and C; Additional file 1: Table S2). The synergistically increased rate of ectopic gene conversion in the *rad27::LEU2 rad59-Y92A* double mutant is consistent with *rad59-Y92A* stimulating HR by a mechanism distinct from the accumulation of replication lesions that results from loss of *RAD27*.

The hyper-rec effects of the *rad59-Y92A* and *srs2::TRP1* alleles are genetically equivalent

Previous work indicating that *rad59-Y92A* decreases spontaneous *RAD51*-independent HR between directly repeated sequences [27] suggests that the stimulation of ectopic gene conversion is not due to accumulation of recombinogenic lesions. Ectopic gene conversion requires Rad51 to work after lesion formation to catalyze the strand invasion that begins the interaction between unlinked sequences that will repair the lesion [40,42]. If

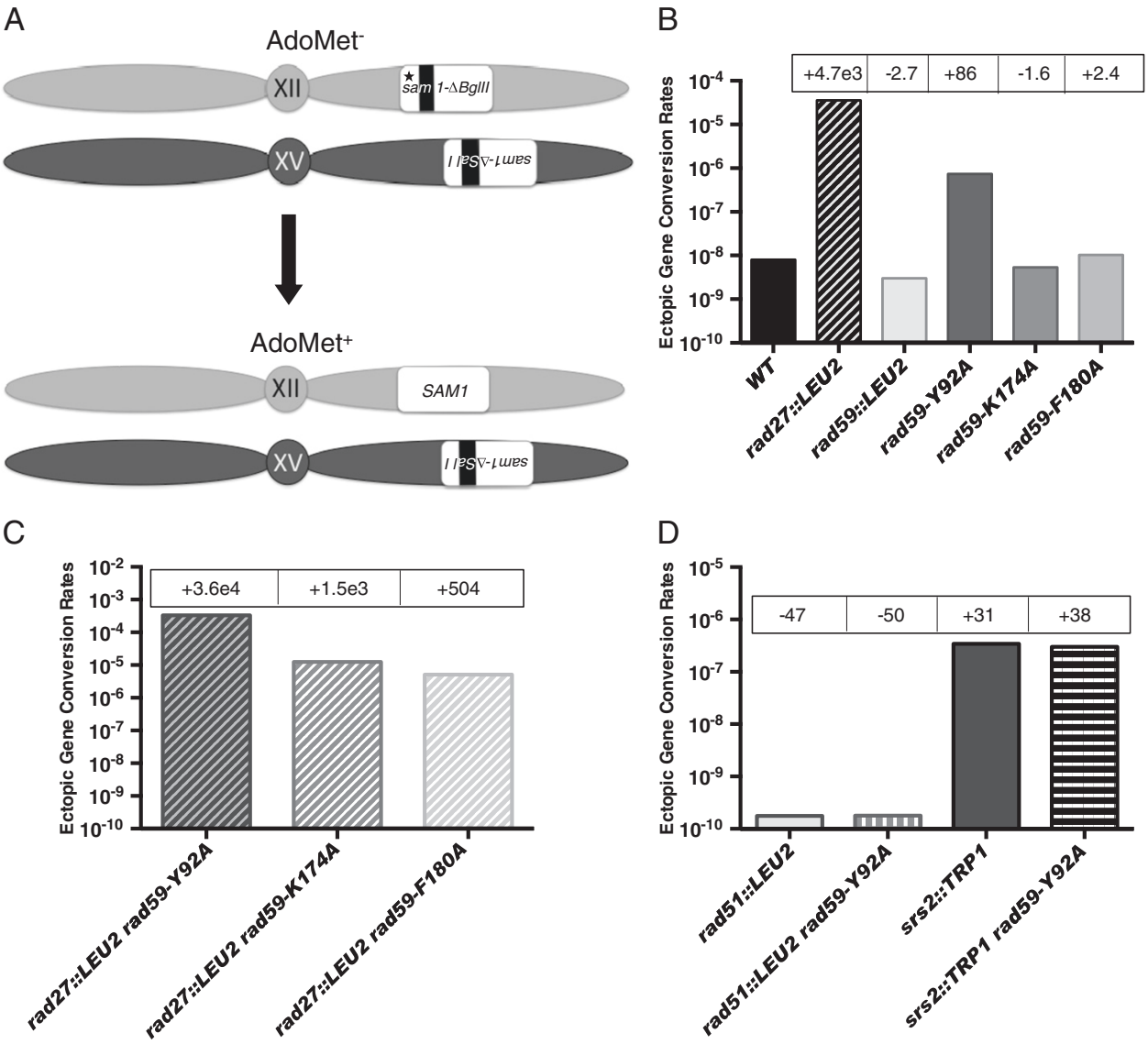

Figure 3 The *rad59* mutant alleles have distinct effects on gene conversion between un-linked repetitive elements in haploid strains. **(A)** The spontaneous ectopic gene conversion system: Haploid strains containing a *sam1-ΔBgl* II-*HOcs* allele at the *SAM1* locus on chromosome XII, a *sam1-ΔSal* I allele at the *HIS3* locus on chromosome XV, and the *sam2::HIS3* allele at the *SAM2* locus on chromosome IV (not pictured) were grown to saturation in YPD supplemented with AdoMet, and plated onto medium lacking AdoMet to select for cells in which a recombination event generates a functional *SAM1* gene and an AdoMet prototrophic cell. The opposite orientations of the *sam1* alleles relative to their centromeres prevents the isolation of single crossovers. Only conversions of the *sam1-ΔBgl* II-*HOcs* allele to wild-type are observed due to the absence of a promoter for the *sam1-ΔSal* I allele. The *sam2::HIS3* allele is missing sufficient information to recombine with *sam1-ΔBgl* II-*HOcs*. Black bars indicate the positions of the mutations. **(B)** Rates of ectopic gene conversion in wild-type and single mutant strains. Rates were determined from a minimum of 10 independent cultures as described in the Methods. Fold decreases (–) and increases (+) from wild-type are indicated in boxes. **(C)** Rates of ectopic gene conversion in *rad27 rad59* double mutant strains. **(D)** Rates of ectopic gene conversion in *rad51::LEU2* and *srs2::TRP1* single mutant, and *rad51::LEU2 rad59-Y92A* and *srs2::TRP1 rad59-Y92A* double mutant strains.

stimulation of HR by *rad59-Y92A* is the result of changes subsequent to Rad51-DNA filament formation, loss of *RAD51* should abolish the stimulatory effect. The rate of ectopic gene conversion in the *rad51::LEU2 rad59-Y92A* double mutant was reduced 50-fold from wild-type, which was nearly identical to the rate in *rad51::LEU2* single mutant cells (Figure 3D; Additional file 1: Table S2). Therefore, stimulation by *rad59-Y92A* requires formation of Rad51-DNA filaments.

Like the *rad59-Y92A* mutation, a null allele of the *SRS2* gene, which encodes a DNA helicase [43] that facilitates the disassembly of Rad51-DNA filaments [36,37], has been shown to stimulate spontaneous gene conversion between non-allelic sequences [44,45]. Consistent with

this, we observed a 31-fold increased rate of spontaneous ectopic gene conversion in an *srs2::TRP1* mutant (Figure 3D; Additional file 1: Table S2). As the effects of *srs2::TRP1* and *rad59-Y92A* were similar we examined ectopic gene conversion in the *srs2 rad59-Y92A* double mutant and observed a 38-fold increase over wild-type that was not significantly different from the rates in the *srs2::TRP1* or *rad59-Y92A* single mutants (Figure 3B and 3D; Additional file 1: Table S2). This indicates that *rad59-Y92A* and *srs2::TRP1* are mutually epistatic.

rad59-Y92A is a dominant mutation that stimulates *RAD51*-dependent HR between homologs

RAD51-dependent HR between sister-chromatids and homologous chromosomes are thought to be the primary mechanisms for rescuing lesions, and supporting viability in *rad27* mutant cells [18-20]. One form of inter-homolog HR that requires *RAD51* is recombination between mutant alleles at the same locus, referred to as heteroallelic recombination [46]. Accordingly, the rate of spontaneous recombination between heteroalleles of the *SAM2* gene was reduced 10.5-fold in the *rad51::LEU2/rad51::LEU2* homozygote (Figure 4B; Additional file 1: Table S2). Consistent with its effect on ectopic gene conversion, loss of *RAD27* increased the rate of heteroallelic recombination 2,400-fold, confirming that accumulation of replication lesions robustly stimulates heteroallelic recombination [18].

Similar to its effect on ectopic gene conversion, we observed that *rad59-Y92A* increased the rate of heteroallelic recombination by 19-fold (Figure 4B; Additional file 1: Table S2). Interestingly, the effect of *rad59-Y92A* was dominant with respect to *RAD59*, as the rate in the *RAD59/rad59-Y92A* heterozygote was not significantly different from that in the *rad59-Y92A/rad59-Y92A* homozygote. Like with ectopic gene conversion, combining the *rad27::LEU2* and *rad59-Y92A* alleles in the *rad27/rad27 rad59-Y92A/rad59-Y92A* double homozygote had a synergistic effect on heteroallelic recombination, increasing the rate 25-fold over that observed in the *rad27::LEU2/rad27::LEU2* homozygote. This astonishing, 59,000-fold increased rate of heteroallelic recombination corresponds to a median frequency of recombination where 85% of the surviving colonies are recombinants.

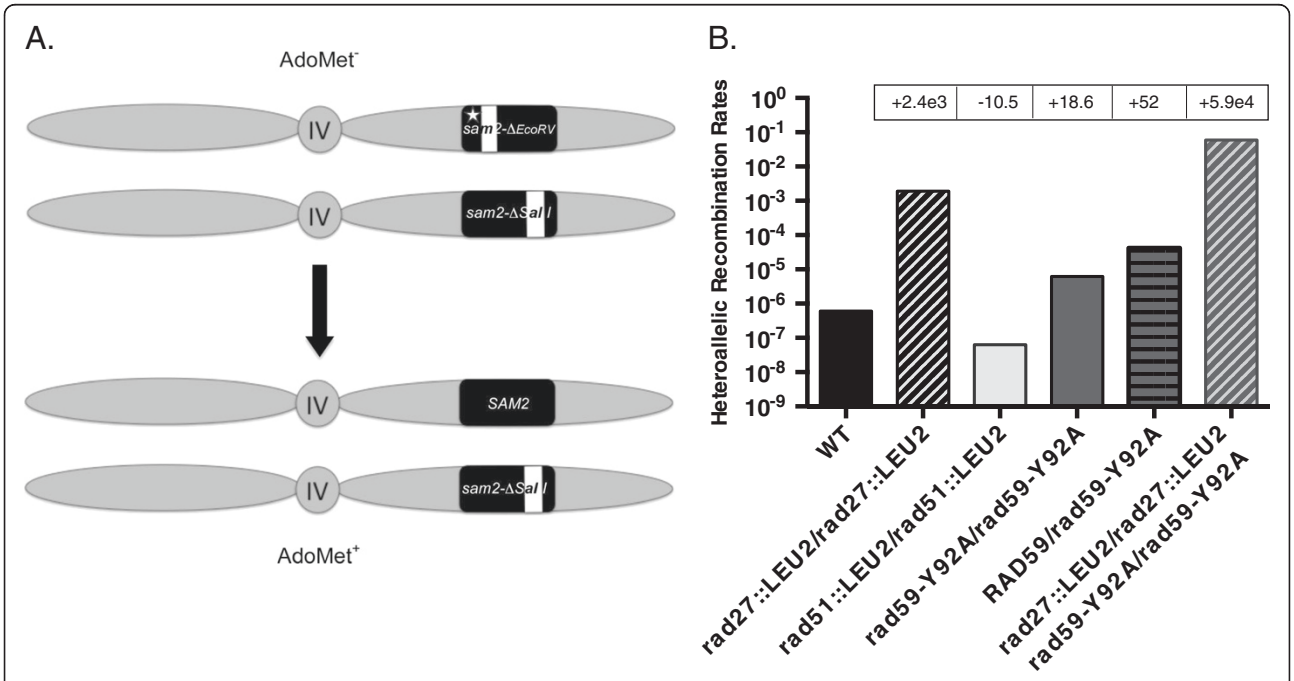

Figure 4 The *rad59-Y92A* mutation has a dominant, hyper-rec effect on hetero-allelic recombination in diploid strains. (A) The spontaneous hetero-allelic recombination system: Diploid strains possessing a *sam2-ΔEcoR* V-*HOcs* allele at the *SAM2* locus on one copy of chromosome IV, a *sam2-ΔSall* allele on the other copy, and a *sam1::LEU2* allele at the *SAM1* locus on both copies of chromosome XII (not pictured) were grown to saturation in YPD medium supplemented with AdoMet, and plated onto medium lacking AdoMet to select for cells in which a recombination event generates a functional *SAM2* gene and an AdoMet prototrophic cell. Either reciprocal or non-reciprocal recombination events between *sam2-ΔEcoR* V-*HOcs* and *sam2-ΔSal* I can generate AdoMet$^+$ recombinants. The *sam1::LEU2* allele is missing sufficient information to recombine with the *sam2* alleles. The white bars indicate the positions of the *sam2* mutations. **(B)** Rates of heteroallelic recombination in wild-type, heterozygous and homozygous mutant strains. Rates were determined from a minimum of 10 independent cultures as described in the Methods. Fold decreases (–) and increases (+) from wild-type indicated in boxes.

The *rad59* alleles do not affect a variety of genome destabilizing processes stimulated by the accumulation of replication lesions

Loss of *RAD27* stimulates a variety of mutagenic and clastogenic events [8,16,18,47,48]. The rate of spontaneous mutation at the *CAN1* locus is greatly increased in *rad27::LEU2* mutant cells [8,18,49]. Characterization of these mutations revealed that the majority are short duplications flanked by short, directly repeated sequences that may be created by multiple HR mechanisms [18]. Our data confirm the previous analyses as we observed a 50-fold increased rate of spontaneous mutation at the *CAN1* locus in a *rad27::LEU2* mutant (Table 2; Additional file 1: Table S2). In contrast, the *rad59::LEU2*, *rad59-Y92A*, *rad59-K174A,* and *rad59-F180A* alleles did not have significant effects on the rate of *CAN1* mutation, nor did the missense alleles have significant effects when combined with the *rad27::LEU2* allele.

Loss of *RAD27* has been previously observed to strongly stimulate unequal sister chromatid recombination (USCR) (Additional file 1: Figure S2) [8,50]. We observed a 47-fold increased rate of USCR in *rad27::LEU2* cells (Table 2; Additional file 1: Table S2), confirming the previous results, while loss of *RAD51* had no significant effect. The *rad59::LEU2*, *rad59-Y92A*, *rad59-K174A,* and *rad59-F180A* alleles did not have significant effects on the rate of USCR, nor did the missense mutations have effects in combination with *rad27::LEU2*, suggesting that *RAD59* does not influence this mechanism of genome rearrangement.

Disrupting lagging strand synthesis by imposing a defect in the processivity of Pol δ, or loss of *RAD27*, was shown previously to substantially increase rates of loss of heterozygosity (LOH) by chromosome loss, and HR between homologs [2,8,10,11,18]. In the present analysis, LOH was examined in diploid strains by simultaneously monitoring changes in the genetic state at three loci on chromosome V (*HXT13*, *CAN1* and *HOM3*) in order to separately determine rates of chromosome loss (reduction to hemizygosity at all three loci), terminal LOH (homozygosity at *HXT13* and *CAN1*), and interstitial LOH (homozygosity at *CAN1*) (Additional file 1: Figure S3; Table 3; Additional file 1: Table S2). Rates of all three events increased substantially in *rad27::LEU2/rad27::LEU2* homozygotes; chromosome loss increased 12-fold, terminal LOH increased 37-fold, and interstitial LOH increased 11-fold, strongly suggesting that replication lesions stimulate HR between homologs when they are repaired, and chromosome loss when they are not. Interestingly, we observed an 18-fold increase in the rate of chromosome loss in *rad51::LEU2/rad51::LEU2* homozygotes, consistent with a requirement for *RAD51* in the rescue of broken chromosomes. In contrast, loss of *RAD51* did not have significant effects on interstitial LOH or terminal LOH, indicating that these inter-chromosomal HR events do not require Rad51.

Table 2 Rates of mutation and unequal sister chromatid recombination in wild-type and mutant haploid strains

Genotype	Mutation rate (10^{-7})	USCR rate (10^{-6})
Wild-type	4.0 (3.8, 7.4) [1]	1.0 (0.8, 1.2) [1]
rad51::LEU2	n.d.	1.4 (1.0, 1.8) [+1.4]
rad59::LEU2	7.5 (6.6, 8.6) [+1.9]	0.82 (0.43, 1.4) [-1.3]
rad59-Y92A	4.4 (3.9, 5.3) [+1.1]	1.3 (1.1, 1.8) [+1.3]
rad59-K174A	3.2 (1.8, 5.5) [-1.3]	1.1 (0.85, 2.1) [+1.1]
rad59-F180A	4.8 (4, 6.9) [+1.2]	0.61 (0.47, 0.95) [-1.6]
rad27::LEU2	200 (90, 590) [+50]	47 (39, 100) [+47]
rad27::LEU2 rad59-Y92A	220 (60, 510) [+55]	39 (25, 99) [+39]
rad27::LEU2 rad59-K174A	130 (110, 190) [+32.5]	38 (33, 53) [+38]
rad27::LEU2 rad59-F180A	190 (110, 500) [+47.5]	60 (49, 120) [+60]

Rates of *CAN1* mutation or USCR were determined from at least 10 independent cultures as described in the Methods. The 95% confidence intervals are in parentheses. Fold decreases (–) and increases (+) from wild-type are in brackets. n.d. – not determined.

As observed above for mutation and USCR (Table 2; Additional file 1: Table S2), the *rad59-Y92A*, *rad59-K174A*, and *rad59-F180A* alleles had no significant effect on the rates of interstitial LOH, terminal LOH, and chromosome loss in the *rad59/rad59* single mutants, or in the double mutant combinations with the *rad27::LEU2* allele (Table 3; Additional file 1: Table S2). Similarly, *rad59::LEU2* had no significant effect on the rates of interstitial LOH and terminal LOH, but conferred a small (two-fold), statistically significant increase in chromosome loss. These data suggest that *RAD59* has little influence on these mechanisms of LOH.

Discussion

We have explored the role of *RAD59* in mediating responses to DNA lesions that accumulate in *rad27::LEU2* mutant cells, and found that it supports multiple, genetically separable functions. Like the *rad59::LEU2* allele, we found that the *rad59-K166A* allele, which alters a lysine in a conserved, putative α-helical domain (Additional file 1: Figure S1B) [27,34,35], results in synthetic lethality when combined with the *rad27::LEU2* allele (Figure 1). In previous experiments, we found that *rad27::LEU2* mutant cells display a profusion of DSBs [8]. As both *rad59::LEU2* and *rad59-K166A* substantially reduce association of Rad52 with DSBs [21], we speculate that a critical reduction in the association of Rad52 with the many DSBs in *rad27::LEU2 rad59::LEU2* and *rad27::LEU2 rad59-K166A* double mutants may inhibit their rescue by HR, and results in a lethal level of chromosome loss. The *rad59-F180A* and *rad59-K174A* alleles, which change conserved residues in the same α-helical domain altered by *rad59-K166A*, may have incrementally less severe effects on association of

Table 3 Rates of loss of heterozygosity in wild-type and mutant diploid strains

Genotype	ILOH rate (10^{-5})	TLOH rate (10^{-4})	CL rate (10^{-5})
Wild-type	2.5 (2.1, 3.1) [1]	0.92 (0.62, 1.2) [1]	3.0 (2.5, 3.9) [1]
rad51::LEU2/rad51::LEU2	1.2 (0.92, 2.5) [−2]	1.3 (0.38, 2) [+1.4]	54 (19, 64) [+18]
rad59::LEU2/rad59::LEU2	1.8 (1.2, 2.9) [−1.4]	1.4 (1.1, 1.9) [+1.5]	6.2 (5.8, 10.2) [+2]
rad59-Y92A/rad59-Y92A	3.2 (2.7, 4.8) [+1.3]	0.95 (0.83, 1.5) [1]	2.5 (2.0, 3.6) [−1.2]
rad59-K174A/rad59-K174A	2.0 (1.3, 3.5) [−1.3]	0.76 (0.40, 1.1) [−1.2]	5.6 (2.9, 8.4) [+1.9]
rad59-F180A/rad59-F180A	3.8 (3.1, 5.1) [+1.5]	0.82 (0.63, 1.7) [−1.1]	3.0 (1.5, 7.9) [1]
rad27::LEU2/rad27::LEU2	28 (25, 64) [+11]	34 (24, 47) [+37]	38 (29, 54) [+13]
rad27::LEU2/rad27::LEU2 rad59-Y92A/rad59-Y92A	28 (13, 56) [+11]	36 (17, 50) [+39]	29 (23, 74) [+9.7]
rad27::LEU2/rad27::LEU2 rad59-K174A/rad59-K174A	26 (22, 55) [+10]	33 (24, 39) [+36]	32 (18, 48) [+11]
rad27::LEU2/rad27::LEU2 rad59-F180A/rad59-F180A	52 (29, 76) [+21]	35 (22, 57) [+38]	57 (18,124) [+19]

Rates of interstitial LOH (ILOH), terminal LOH (TLOH), and chromosome loss (CL) from a minimum of 12 independent cultures were determined as described in the Methods. The 95% confidence intervals are in parentheses. Fold decreases (−) and increases (+) from wild-type are in brackets.

Rad52 with DSBs. This may result in their serially reduced inhibition of repair of replication-induced DSBs by HR (Figure 3C; Additional file 1: Table S2) and commensurate effects on growth (Table 1; Additional file 1: Table S2) when combined with *rad27*. An accumulation of *rad27::LEU2 rad59-F180A* double mutant cells in the G2 phase of the cell cycle, as compared to *rad27::LEU2* single mutant or *rad27::LEU2 rad59-K174A* double mutant cells is consistent with more deficient repair of replication-induced DSBs by HR (Figure 3). This further supports the notion that *RAD59* promotes the survival of *rad27::LEU2* mutant cells by facilitating the rescue of replication lesions by HR. Recently, *RAD59* has been shown to be required for the viability of DNA ligase I-deficient mutants, verifying the requirement for this factor in accommodating to incomplete DNA replication [51].

In striking contrast to the other *rad59* alleles, *rad59-Y92A* stimulated HR (Figure 3B; Figure 4B). This hyper-recombinogenic effect was distinct from that caused by *rad27* as it was not accompanied by significant effects on doubling time (Table 1), cell cycle profile (Figure 2), mutation (Table 2), unequal sister chromatid exchange, or LOH (Table 3), suggesting that *rad59-Y92A* does not cause an accumulation of replication lesions. The observation that the stimulatory effect of *rad59-Y92A* was completely suppressed by a null allele of *RAD51*, and was mutually epistatic with a null allele of *SRS2* (Figure 3D), suggests that *rad59-Y92A* may increase HR by increasing the stability of Rad51-DNA filaments, perhaps by changing its interaction with Rad51 (24). An increase in DSBs combined with an increase in the stability of Rad51 filaments at the DSBs may underlay the synergistically increased rates of HR observed in *rad27 rad59-Y92A* double mutants (Figures 3C and 4B). However, since Rad59 also interacts with RPA [52] and RSC [53], the increase in HR observed in *rad59-Y92A* mutant cells may also involve changes in additional processes.

While our results support a prominent role for *RAD59*-dependent HR in the repair of replication lesions in *rad27::LEU2* mutants, HR mechanisms that do not depend on *RAD59* were also strongly stimulated in *rad27::LEU2* mutants. In particular, inter-chromosomal HR leading to interstitial and terminal LOH events was strongly stimulated by *rad27*, but was unaffected by the *rad59* alleles (Table 3). Inter-chromosomal HR leading to LOH is thought to occur by break-induced replication (BIR) [54]. BIR has been proposed to utilize a single-ended DSB on one homolog to generate a replication fork-like intermediate with the unbroken homolog that may potentially proceed until reaching the end of the donor chromosome (Additional file 1: Figure S4A) [22]. In contrast, *RAD59*-dependent heteroallelic recombination is thought to utilize a double-ended DSB where both ends are rescued, either through concerted interactions with the unbroken homolog, or through the first end interacting with the homolog followed by the second end annealing with the first after gaining sequences copied from the unbroken homolog (Additional; file 1: Figure S4B). The stimulation of both mechanisms of HR between homologs suggests that loss of *RAD27* leads to the accumulation of both single- and double-ended DSBs. DSBs may arise when the failure to remove flaps on the 5′ ends of Okazaki fragments leads to accumulation of nicks on newly replicated lagging strands (Figure 5). Persistence of these nicks into the subsequent cell cycle will leave discontinuities on the template for leading strand synthesis that will stall replication and form single-ended DSBs. If a second replication fork from an adjacent replicon collides with the first stalled fork, a double-ended DSB can arise. A genome-wide increase in replication-induced DSB formation, like that induced by many chemotherapeutic agents, would therefore require a robust response by the HR apparatus to prevent chromosome loss, potentially explaining the critical role of HR in determining sensitivity to these drugs in humans [55,56].

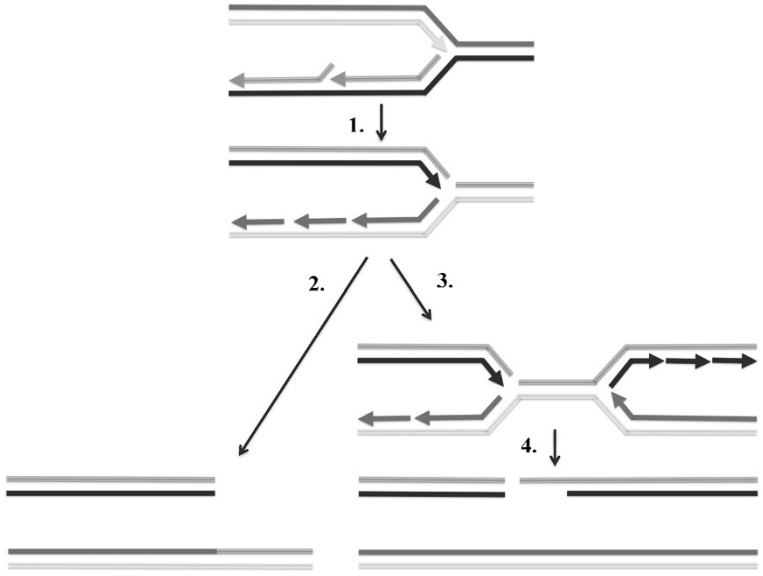

Figure 5 Models for initiation of *RAD51*- and *RAD59*-dependent and –independent HR by defective lagging strand synthesis. 1.) Accumulation of daughter strand nicks in the absence of Rad27 nuclease causes replication fork stalling during the next S phase when the lagging strand becomes the template for leading strand synthesis and the replication fork encounters the discontinuity. 2.) The stalled fork is converted into an intact chromatid and a single-ended DSB. The single-ended DSB becomes a substrate for *RAD51*- and *RAD59*-independent HR mechanisms, such as interstitial and terminal LOH (Additional file 1: Figure S3). 3.) The replication fork from an upstream replicon converges with the previously stalled fork. 4.) Converged forks are converted into an intact chromatid and a double-ended DSB. The double-ended DSB becomes a substrate for *RAD51*- and *RAD59*-dependent HR mechanisms, such as ectopic gene conversion and heteroallelic recombination (Figures 3A and 4A).

Conclusions

RAD59 encodes one of several homologous recombination (HR) factors required for viability of budding yeast cells lacking the DNA replication factor, Rad27. This demonstrates that the HR apparatus is required to prevent the lethal effects of dysfunctional replication, but no link between a specific HR mechanism and survival has been previously established. In this analysis, we show that two *rad59* alleles that diminish association of Rad52 with double-strand breaks are synthetically lethal with *rad27*, while two others coordinately reduce *RAD51*-dependent HR and growth, thus linking *RAD51*-dependent repair with survival. Another allele stimulates HR by stabilizing Rad51-DNA filaments. Therefore, Rad59 influences the repair of replication lesions by HR through its interactions with multiple HR factors. We speculate that the massive increase in replication failure genome-wide that results from loss of Rad27 may be similar to that caused by chemotherapeutic agents in human cells, potentially explaining why the HR apparatus is critical in determining sensitivity to these drugs.

Methods

Strains

All strains used in this study were isogenic and are listed in Additional file 1: Table S1. Standard techniques for yeast strain construction and growth were used [57]. Construction of the *rad27::LEU2, rad51::LEU2, rad59::LEU2, rad59-Y92A, rad59-K166A, rad59-K174A, rad59-F180A* and *srs2::TRP1* alleles have been described previously [27,58-60]. The *rad27::LEU2* allele can be followed in crosses by PCR, using the forward primer 5′-GCG TTG ACA GCA TAC ATT-3′, and reverse primer 5′-CGT ACA AAC CAA ATG CGG-3′. The *rad59::LEU2* allele is followed by PCR using the forward primer 5′-GCC ACA GTT TGG CAA GGG-3′, and the reverse primer 5′-GGG TTT GTT GCC ATC TGC G-3′. The *rad59* missense alleles were followed in crosses by allele-specific PCR [27]. Unique forward primers were used to detect *rad59-Y92A* (5′-GCT AAT GAA ACA TTC GGG GC-3′), *rad59-K166A* (5′-AAT GTT ATA ACA GGT CGA AAG C-3′), *rad59-K174A* (5′-AAG GGT TAC GTA GAG GAG AAG-3′), and *rad59-F180A* (5′-AAG AAG GCG TTA TTG AGC GC-3′). All allele-specific PCRs use the same reverse primer (5′-TAT ATA AGT ACG TGA GAT CTA TTT G-3′). Presence of the *rad59-K174A* allele is scored by digesting the PCR product with *MseI* restriction endonuclease. DNA was purified for PCR analysis using a standard method [61].

Synthetic lethality

Diploid yeast strains heterozygous for each of the *rad59* alleles (*rad59/RAD59*) and the *rad27::LEU2* allele (*rad27::LEU2/RAD27*) were sporulated and dissected. After 72 h, five representative tetrads from each diploid were selected. The presence of *rad27* and *rad59* mutant alleles in each of

the colonies that arose from the spores was scored using PCR as described above.

Doubling time

At least 10, five-milliliter YPD (1% yeast extract, 2% peptone, 2% dextrose) cultures were inoculated with colonies arising from the spores of freshly dissected tetrads and grown overnight at 30°. These were sub-cultured into Klett tubes containing five milliliters of YPD medium that were incubated at 30° while shaking. Cell density was measured by monitoring culture turbidity with a Klett-Summerson colorimeter each hour over a 10 h period. Doubling times were calculated using a standard algorithm [62]. The 95% confidence intervals and Mann–Whitney values were determined using the Prism statistics package (GraphPad, La Jolla, CA).

Flow cytometry

At least five, five-milliliter YPD cultures were inoculated with colonies arising from freshly dissected tetrads and grown overnight at 30°. Overnight cultures were sub-cultured into five milliliters of YPD medium and grown to mid-log phase at 30° defined by growth curve using a Klett-Summerson colorimeter. Cells were processed for flow cytometry using the following adaptation of a published method [63]. The cell density was determined by hemacytometer count and aliquots containing 10^7 cells were pelleted, resuspended in 70% ice-cold ethanol, and fixed while rotating at 4° overnight. Fixed cells were pelleted, resuspended in 1 ml of citrate buffer (50 mM Na citrate, pH 7.2), and sonicated (Misonix 3000, Farmingdale, NY). Sonicated cells were pelleted, resuspended in citrate buffer and treated with 25 μl of 10 mg/ml RNase A, at 50° for one h, followed by treatment with 50 μl of 20 mg/ml Proteinase K and incubation at 50° for one h. Cells were pelleted and resuspended in 1 ml of citrate buffer, and either rotated overnight at 4°, or stained immediately by adding 16 μl of 1 mg/ml propidium iodide and rotating for 45 min at room temperature in the dark before processing by flow cytometry (Beckman Coulter CyAn ADP 9color, Miami FL). Fractions of cells in the G1, S and G2/M phases of the cell cycle were determined using FlowJo v.7.6.5 image processing software (Tree Star, Ashland, OR). The ratio of cells in G1 vs. S + G2/M were calculated for each trial and the median value for each strain used for comparing cell cycle distributions in different strains. The Mann–Whitney test was used to assess the statistical significance of differences between strains.

Spontaneous ectopic gene conversion

Spontaneous ectopic gene conversion in haploid strains was assayed as described previously [64], but using substrates described in a separate analysis [41]. All strains contained the *sam1-ΔBgl* II-*HOcs* allele at the *SAM1* locus on chromosome XII, the *sam1-ΔSal* I allele adjacent to the *HIS3* locus on chromosome XV, and a *HIS3* gene replacing the *SAM2* coding sequence at the *SAM2* locus (*sam2::HIS3*) on chromosome IV. The *sam1-ΔBgl* II-HOcs allele has a 117 bp fragment of the *MAT* locus disrupting the *Bgl* II site in the *SAM1* coding sequence, while the *sam1-ΔSal* I allele has a 4 bp insertion at the *Sal* I site [41]. The *sam1-ΔSal* I allele lacks a promoter, preventing conversion events at this locus from generating AdoMet$^+$ recombinants. The *sam1-ΔBgl* II-HOcs and *sam1-ΔSal* I alleles are also in opposite orientations relative to their centromeres, preventing the isolation of single crossover recombinants.

At least ten freshly dissected haploid segregants of each strain were inoculated into five-milliliter YPD cultures supplemented with 100 μg/ml of *S*-adenosylmethionine (AdoMet) and grown to saturation at 30°. Appropriate dilutions of each culture were plated onto YPD + AdoMet plates to determine the number of viable cells, and onto YPD plates lacking AdoMet to determine the number of AdoMet prototrophic recombinants.

All rates were determined by the method of the median [65]. Rates and 95% confidence intervals were calculated as described previously [66].

Spontaneous hetero-allelic recombination

Rates of spontaneous hetero-allelic recombination were determined as for ectopic gene conversion except that different substrates were used in diploid cells. All strains contained the *sam2-ΔEcoR* V-*HOcs* allele at the *SAM2* locus on one copy of chromosome IV, the *sam2-ΔSal* I allele on the other, and a *LEU2* marker replacing the *SAM1* coding sequence at the *SAM1* locus on both copies of chromosome XII. The *sam2-ΔEcoR* V-*HOcs* allele has a 117 bp fragment of the *MAT* locus disrupting the *EcoR* V site, while the *sam2-ΔSal* I allele has a 4 bp insertion disrupting the *Sal* I site [41].

Mutation rate

Rates of mutation at the *CAN1* locus were examined using a previously published assay [8,10,18]. At least ten freshly dissected segregants were used to inoculate one-milliliter YPD cultures that were grown to saturation at 30°. Appropriate dilutions were plated onto YPD to determine viability and synthetic medium lacking arginine but containing 60 μg/ml of canavanine to select for mutants.

Unequal sister chromatid recombination (USCR)

Rates of USCR were determined using a previously published assay [8,10,67]. At least ten freshly dissected segregants containing the USCE construct at the *TRP1* locus on chromosome IV and the *his3Δ200* allele at the

HIS3 locus on chromosome XV, were struck out to single colonies on YPD. After three days of growth at 30°, single colonies were used to inoculate one-milliliter YPD cultures, and grown to saturation at 30°. Appropriate dilutions were plated onto YPD to assess viability and onto medium lacking histidine to determine the number of histidine prototrophic recombinants.

Loss of heterozygosity (LOH)

Rates of spontaneous LOH by three different mechanisms were assessed using a previously published assay [8]. Freshly dissected haploid segregants containing either the *hxt13::URA3, CAN1,* and *HOM3* alleles, or the *HXT13, can1-100,* and *hom3-10* alleles on chromosome V were crossed and the resulting diploids struck out to single colonies on YPD. At least 12 independent colonies were inoculated into one-milliliter YPD liquid cultures and grown to saturation at 30°. Appropriate dilutions were plated onto YPD for viability and synthetic medium lacking arginine, but containing 60 μg/ml of canavanine to select for clones resistant to canavanine. After three days of growth at 30° canavanine-resistant (Can^R) colonies were replica plated onto synthetic medium lacking either uracil or threonine to assay for the presence of the *hxt13::URA3* (Ura^+) and *HOM3* (Thr^+) alleles, respectively. Rates of interstitial LOH, terminal LOH, and CL were determined from the number of $Ura^+Can^RThr^+$, $Ura^-Can^RThr^+$, and $Ura^-Can^RThr^-$ recombinant colonies, respectively.

Modeling the Rad59 protein

The crystal structure of the N-terminus of human Rad52 [34] was obtained from the RSCB Protein Data Bank (http://www.rcsb.org/pdb/). This structure was imaged using the molecular modeling program, SYBYL, and the amino acids corresponding to those mutated in the *rad59* missense alleles were identified, and highlighted.

Availability of supporting data

The data sets supporting the results of this article are included within the article and in Additional file 1.

Additional file

Additional file 1: Table S1. *Saccharomyces cerevisiae* strains used in this study. **Table S2.** Summary of quantitative data. **Figure S1. A.** Multiple amino acid sequence alignment of ScRad59 with ScRad52 and HsRad52. **B.** Molecular modeling of the proteins encoded by the *rad59* missense alleles demonstrates that Rad59-Y92A is in a different structural motif. **Figure S2.** The unequal sister chromatid recombination (USCR) assay for measuring spontaneous homologous recombination between sister chromatids in haploid yeast. **Figure S3.** The loss of heterozygosity assay for measuring spontaneous Rad51-independent homologous recombination. **Figure S4.** LOH is the recombination product of a single-ended DSB, whereas HAR results from repair of a double-ended DSB. **A)** LOH results from the repair of a single-ended DSB by HR. **B)** HAR results from the repair of a double-ended DSB by HR.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LL carried out the synthetic lethality experiments, LOH genetic studies, flow cytometric analysis, sequence alignment, designed the figures and tables, and drafted the manuscript. GM performed the growth, mutation and USCR rate studies. SO assisted with the synthetic lethality and LOH experiments. BF contributed to the LOH experiments. AB conceived of the study, designed and carried out the ectopic gene conversion and hetero-allelic recombination analyses, and helped draft the manuscript. All authors read and approved the final manuscript.

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